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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/DK96/00390 <b>(22) International Filing Date:</b> 13 September 1996 (13.09.96) <b>(30) Priority Data:</b> 1042/95 19 September 1995 (19.09.95) DK 1317/95 23 November 1995 (23.11.95) DK <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DAMHUS, Ture [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). SCHNEIDER, Palle [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). CONRAD, Lars, Sparre [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). EBDROP, Søren [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). SPILE, Nickie, Inger [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> STAIN BLEACHING  <b>(57) Abstract</b>  The invention relates to a process for bleaching stains present on fabric, comprising contacting, in an aqueous medium, the fabric with a phenol oxidizing enzyme system and a mediator for a sufficient period of time.		

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**STAIN BLEACHING****FIELD OF INVENTION**

5           The present invention relates to a process for removing coloured stains present on fabric at low washing temperatures.

**BACKGROUND ART**

10

When washing clothes and other types of fabric there are some coloured stains which are problematic to remove: These stains typically originate from red wine, fruit such as black currant, cherry, strawberry and tomato (in particular ketchup and spaghetti sauce), vegetables such as carrots and beetroot, 15           tea, coffee, spices such as curry and paprika, grass, or ball pens/ink.

Commercial detergents normally include bleaching agents to solve the above mentioned problem. Traditionally, 20           bleaching agents incorporated in detergent compositions are compounds which are precursors of hydrogen peroxide; hydrogen peroxide is then formed in the course of the washing procedure. Perborates and percarbonates are the most important examples of such hydrogen peroxide precursors. Perborate and percarbonate 25           may also be combined with a peracid-forming bleach activator such as TAED (tetraacetylenethylenediamine) or NOBS (nonanoyloxybenzenesulfonate).

These traditional bleaching systems function quite satisfactorily at high temperatures, the optimum operation 30           temperature of these systems being typically above 60°C.

An important challenge during the last years has been to provide efficient bleaching systems which function at low temperatures (10°C - 50°C).

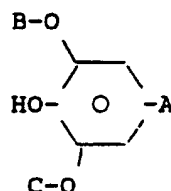
In WO 89/09813 it is suggested to remove stains by 35           using peroxidase. The process has an effect but a more efficient

stain bleaching system useful at low temperatures is still needed.

# SUMMARY OF THE INVENTION

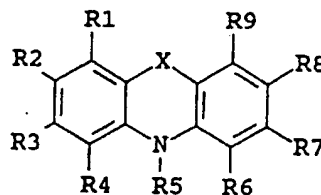
It has now surprisingly been found possible to create a stain bleaching process which effectively removes stains at low temperatures, said process comprising contacting, in an aqueous medium, the fabric with a phenol oxidizing enzyme system and a mediator for a sufficient period of time, wherein said mediator can be described by one of the following formulas:

I:



in which formula A is a group such as -CO-X, -CH=CH-X, -CH=CH-CH=CH-X, -CH=CH-CO-X, -SO<sub>2</sub>-X, -PO-XZ, in which X and Z may be identical or different and selected from the group consisting of -H, -OH, -C<sub>n</sub>H<sub>2n+1</sub>, -OC<sub>n</sub>H<sub>2n+1</sub> and -N-EF, in which E and F may be identical or different and selected from the group consisting of -H and C<sub>n</sub>H<sub>2n+1</sub>; 1 ≤ n ≤ 5; and B and C may be the same or different and selected from C<sub>m</sub>H<sub>2m+1</sub>; 1 ≤ m ≤ 5; or

II:



in which formula X represents (-O-) or (-S-), and the

substituent groups  $R^1$ - $R^9$ , which may be identical or different, independently represents any of the following radicals: hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl,  $C_1$ - $C_{14}$ -alkyl,  $C_1$ - $C_5$ -alkoxy, carbonyl- $C_1$ - $C_5$ -alkyl, aryl- $C_1$ - $C_5$ -alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group  $R^{10}$ ; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups  $R^{10}$ ; and which  $C_1$ - $C_{14}$ -alkyl,  $C_1$ - $C_5$ -alkoxy, carbonyl- $C_1$ - $C_5$ -alkyl, and aryl- $C_1$ - $C_5$ -alkyl groups may be saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups  $R^{10}$ ;

which substituent group  $R^{10}$  represents any of the following radicals: halogen, hydroxy, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperazinyl, pyrrolidino,  $C_1$ - $C_5$ -alkyl,  $C_1$ - $C_5$ -alkoxy; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy,  $C_1$ - $C_5$ -alkyl,  $C_1$ - $C_5$ -alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and which  $C_1$ - $C_5$ -alkyl, and  $C_1$ - $C_5$ -alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl;

or in which general formula two of the substituent groups  $R^1$ - $R^9$  may together form a group -B-, in which B represents any of the following the groups:  $(-CHR^{10}-N=N-)$ ,  $(-CH=CH-)_n$ ,  $(-CH=N-)_n$  or  $(-N=CR^{10}-NR^{11}-)$ , in which groups n represents an integer of from 1 to 3,  $R^{10}$  is a substituent group as defined

above and  $R^{11}$  is defined as  $R^{10}$ .

#### DETAILED DESCRIPTION OF THE INVENTION

5           According to the present invention it may be possible to remove coloured stains present on fabric at low washing temperatures, in particular at washing temperatures of from 5°C to 50°C, preferably at washing temperatures of from 10°C to 40°C, more preferably at temperatures of from 10°C to 30°C, even more  
10           preferably at temperatures of from 10°C to 20°C, by using a bleaching process comprising contacting, in an aqueous medium, the fabric with a phenol oxidizing enzyme and a mediator for a sufficient period of time.

          Stains which may be removed according to the present  
15           invention typically originate from for example spices such as curry and paprika or vegetables/fruits, e.g., carrots.

          The bleaching time for removing the stain(s) may vary; the fabric may be soaked for one or two days or the bleaching may be performed within a shorter period, typically for a period  
20           of 1 to 90 minutes, preferably for a period of 1 to 30 minutes.

#### Phenol Oxidizing Enzymes

          By the term "a phenol oxidizing enzyme" is meant an enzyme, which by using hydrogen peroxide or molecular oxygen, is  
25           capable of oxidizing organic compounds containing phenolic groups. Examples of such enzymes are peroxidases and oxidases.

          If the phenol oxidizing enzyme requires a source of hydrogen peroxide, the source may be hydrogen peroxide or a hydrogen peroxide precursor for in situ production of hydrogen  
30           peroxide, e.g., percarbonate or perborate, or a hydrogen peroxide generating enzyme system, e.g., an oxidase and a substrate for the oxidase, or an amino acid oxidase and a suitable amino acid, or a peroxycarboxylic acid or a salt thereof. Hydrogen peroxide may be added at the beginning of or  
35           during the process, e.g., in a concentration corresponding to



0.001-25 mM H<sub>2</sub>O<sub>2</sub>.

If the phenol oxidizing enzyme requires molecular oxygen, molecular oxygen from the atmosphere will usually be present in sufficient quantity.

5 In the context of the present invention the enzyme of the phenol oxidizing enzyme may be an enzyme possessing peroxidase activity or a laccase or a laccase related enzyme as described below.

10 According to the invention the concentration of the phenol oxidizing enzyme in the aqueous medium where the stain bleaching of the fabric is taking place, may be of from 0.001-100 mg of enzyme protein per liter, in particular of from 0.01-50 mg of enzyme protein per liter, even more preferably of from 0.1-10 mg of enzyme protein per liter.

15

#### Peroxidases and Compounds possessing Peroxidase Activity

An enzyme exhibiting peroxidase activity may be any peroxidase enzyme comprised by the enzyme classification (EC 1.11.1.7), or any fragment derived therefrom, exhibiting  
20 peroxidase activity, or synthetic or semisynthetic derivatives thereof (e.g. porphyrin ring systems or microperoxidases, cf. e.g. US 4,077,768, EP 537,381, WO 91/05858 and WO 92/16634).

Preferably, the peroxidase employed in the method of the invention is producible by plants (e.g. horseradish or  
25 soybean peroxidase) or microorganisms such as fungi or bacteria. Some preferred fungi include strains belonging to the subdivision Deuteromycotina, class Hyphomycetes, e.g. *Fusarium*, *Humicola*, *Trichoderma*, *Myrothecium*, *Verticillium*, *Arthromyces*, *Caldariomyces*, *Ulocladium*, *Embellisia*, *Cladosporium* or *Dre-*  
30 *schlera*, in particular *Fusarium oxysporum* (DSM 2672), *Humicola insolens*, *Trichoderma resii*, *Myrothecium verrucaria* (IFO 6113), *Verticillium alboatrum*, *Verticillium dahlie*, *Arthromyces ramosus* (FERM P-7754), *Caldariomyces fumago*, *Ulocladium chartarum*, *Embellisia alli* or *Dreschlera halodes*.

35 Other preferred fungi include strains belonging to the subdivision Basidiomycotina, class Basidiomycetes, e.g.

*Coprinus*, *Phanerochaete*, *Coriolus* or *Trametes*, in particular *Coprinus cinereus* f. *microsporus* (IFO 8371), *Coprinus macrorrhizus*, *Phanerochaete chrysosporium* (e.g. NA-12) or *Trametes* (previously called *Polyporus*), e.g. *T. versicolor* (e.g. PR4 28-A).

Further preferred fungi include strains belonging to the subdivision Zygomycotina, class Mycoraceae, e.g. *Rhizopus* or *Mucor*, in particular *Mucor hiemalis*.

Some preferred bacteria include strains of the order Actinomycetales, e.g. *Streptomyces spheroides* (ATCC 23965), *Streptomyces thermoviolaceus* (IFO 12382) or *Streptoverticillium verticillium* ssp. *verticillium*.

Other preferred bacteria include *Bacillus pumilus* (ATCC 12905), *Bacillus stearothermophilus*, *Rhodobacter sphaeroides*, *Rhodomonas palustri*, *Streptococcus lactis*, *Pseudomonas putrefaciens* (ATCC 15958) or *Pseudomonas fluorescens* (NRRL B-11).

Further preferred bacteria include strains belonging to *Myxococcus*, e.g. *M. virescens*.

The peroxidase may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said peroxidase as well as DNA sequences encoding functions permitting the expression of the DNA sequence encoding the peroxidase, in a culture medium under conditions permitting the expression of the peroxidase and recovering the peroxidase from the culture.

Particularly, a recombinantly produced peroxidase is a peroxidase derived from a *Coprinus* sp., in particular *C. macrorrhizus* or *C. cinereus* according to WO 92/16634, or a variant thereof, e.g., a variant as described in WO 93/24618 and WO 95/10602.

In the context of this invention, compounds possessing peroxidase activity comprise peroxidase enzymes and peroxidase active fragments derived from cytochromes, haemoglobin or peroxidase enzymes, and synthetic or semisynthetic derivatives

thereof, e.g., iron porphyrins, and iron phthalocyanine and derivatives thereof.

#### Determination of Peroxidase Activity (PODU)

5           1 peroxidase unit (PODU) is the amount of enzyme that catalyzes the conversion of 1  $\mu$ mole hydrogen peroxide per minute at the following analytical conditions: 0.88 mM hydrogen peroxide, 1.67 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), 0.1 M phosphate buffer, pH 7.0, incubated at 30°C,  
10 photometrically followed at 418 nm.

#### Laccase and Laccase Related Enzymes

In the context of this invention, laccases and laccase related enzymes comprise any laccase enzyme comprised by the  
15 enzyme classification (EC 1.10.3.2), any catechol oxidase enzyme comprised by the enzyme classification (EC 1.10.3.1), any bilirubin oxidase enzyme comprised by the enzyme classification (EC 1.3.3.5) or any monophenol monooxygenase enzyme comprised by the enzyme classification (EC 1.14.18.1).

20           The above mentioned enzymes may be derived from plants, bacteria or fungi (including filamentous fungi and yeasts) and suitable examples include a laccase derivable from a strain of *Aspergillus*, *Neurospora*, e.g., *N. crassa*, *Podospora*, *Botrytis*, *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, e.g.,  
25 *T. villosa* and *T. versicolor*, *Rhizoctonia*, e.g., *R. solani*, *Coprinus*, e.g., *C. plicatilis* and *C. cinereus*, *Psatyrella*, *Myceliophthora*, e.g., *M. thermophila*, *Schytalidium*, *Polyporus*, e.g., *P. pinsitus*, *Phlebia*, e.g., *P. radita* (WO 92/01046), or *Coriolus*, e.g., *C.hirsutus* (JP 2-238885).

30           The laccase or the laccase related enzyme may furthermore be one which is producible by a method comprising cultivating a host cell transformed with a recombinant DNA vector which carries a DNA sequence encoding said laccase as well as DNA sequences encoding functions permitting the  
35 expression of the DNA sequence encoding the laccase, in a cul-

ture medium under conditions permitting the expression of the laccase enzyme, and recovering the laccase from the culture.

#### Determination of Laccase Activity (LACU)

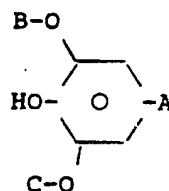
5 Laccase activity is determined from the oxidation of syringaldazin under aerobic conditions. The violet colour produced is photometered at 530 nm. The analytical conditions are 19  $\mu$ M syringaldazin, 23.2 mM acetate buffer, pH 5.5, 30°C, 1 min. reaction time.

10 1 laccase unit (LACU) is the amount of enzyme that catalyses the conversion of 1.0  $\mu$ mole syringaldazin per minute at these conditions.

#### Mediators

15 According to the present invention a mediator is any compound that enhances the bleaching process. The enhancing agent will typically be an organic compound, e.g., an organic compound described by one of the following formulas:

20



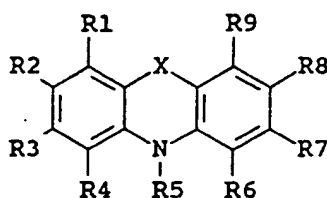
25

in which formula A is a group such as -CO-X, -CH=CH-X, -CH=CH-  
 30 CH=CH-X, -CH=CH-CO-X, -SO<sub>2</sub>-X, -PO-XZ, in which X and Z may be identical or different and selected from the group consisting of -H, -OH, -C<sub>n</sub>H<sub>2n+1</sub>, -OC<sub>n</sub>H<sub>2n+1</sub> and -N-EF, in which E and F may be identical or different and selected from the group consisting of -H and C<sub>n</sub>H<sub>2n+1</sub>; 1 ≤ n ≤ 5; and B and C may be the same or  
 35 different and selected from C<sub>m</sub>H<sub>2m+1</sub>; 1 ≤ m ≤ 5.

In the above mentioned formula A may be placed meta to the hydroxy group instead of being placed in the para-position as shown.

In particular embodiments, the mediator is acetosyringone, syringaldehyde, methylsyringate, syringic acid, ethylsyringate, propylsyringate, butylsyringate, hexylsyringate, octylsyringate or ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate.

The mediator used in the present invention may also be described by the following formula:



15

in which formula X represents (-O-) or (-S-), and the substituent groups  $R^1$ - $R^9$ , which may be identical or different, independently represents any of the following radicals:  
 20 hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl,  $C_1$ - $C_{14}$ -alkyl,  $C_1$ - $C_5$ -alkoxy, carbonyl- $C_1$ - $C_5$ -alkyl, aryl- $C_1$ - $C_5$ -alkyl; which carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or  
 25 substituted once or twice with a substituent group  $R^{10}$ ; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups  $R^{10}$ ; and which  $C_1$ - $C_{14}$ -alkyl,  $C_1$ - $C_5$ -alkoxy, carbonyl- $C_1$ - $C_5$ -alkyl, and aryl- $C_1$ - $C_5$ -alkyl groups may be saturated or unsaturated, branched or unbranched, and may  
 30 furthermore be unsubstituted or substituted with one or more substituent groups  $R^{10}$ ;

which substituent group  $R^{10}$  represents any of the following radicals: halogen, hydroxy, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof,  
 35 sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperaziny, pyrrolidino,  $C_1$ - $C_5$ -alkyl,  $C_1$ - $C_5$ -alkoxy; which

carbamoyl, sulfamoyl, and amino groups may furthermore be unsubstituted or substituted once or twice with hydroxy, C<sub>1</sub>-C<sub>5</sub>-alkyl, C<sub>1</sub>-C<sub>5</sub>-alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and which C<sub>1</sub>-C<sub>5</sub>-alkyl, and C<sub>1</sub>-C<sub>5</sub>-alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl;

or in which general formula two of the substituent groups R<sup>1</sup>-R<sup>9</sup> may together form a group -B-, in which B represents any of the following the groups: (-CHR<sup>10</sup>-N=N-), (-CH=CH-)<sub>n</sub>, (-CH=N-)<sub>n</sub> or (-N=CR<sup>10</sup>-NR<sup>11</sup>-), in which groups n represents an integer of from 1 to 3, R<sup>10</sup> is a substituent group as defined above and R<sup>11</sup> is defined as R<sup>10</sup>.

In particular embodiments, the mediator is 10-methylphenothiazine, 10-phenothiazine-propionic acid, N-hydroxy-succinimide-10-phenothiazine-propionate, 10-ethyl-4-phenothiazine-carboxylic acid, 10-ethylphenothiazine, 10-propylphenothiazine, 10-isopropylphenothiazine, methyl-10-phenothiazinepropionate, 10-phenylphenothiazine, 10-allylphenothiazine, 10-(3-(4-methyl-1-piperazinyl)propyl)phenothiazine, 10-(2-pyrrolidinoethyl)phenothiazine, chlorpromazine, 2-chloro-10-methylphenothiazine, 2-acetyl-10-methylphenothiazine, 4-carboxy-10-phenothiazine, 10-methylphenoxazine, 10-ethylphenoxazine, 10-phenoxazine-propionic acid or 4-carboxy-10-phenoxazine-propionic acid.

The mediator of the invention may be present in concentrations of from 0.01 to 5000 μM, preferably in concentrations of from 0.1 to 1000 μM, even more preferably in concentrations of from 1 to 500 μM.

### Preparation of Mediators

The mediators described in the present application may be prepared using methods well known to those skilled in the art; some of the mediators are also commercially available.

- 5           Some of the mediators may be obtained from Sigma-Aldrich, Janssen Chimica, Kodak, Tokyo Kasai Organic Chemicals, Daiichi Pure Chemicals Co. or Boehringer Mannheim.

- N-methylated derivatives of phenothiazine and phenoxazine may be prepared by methylation with methyl iodide as  
10 described by Cornel Bodea and Ioan Silberg in "Recent Advances in the Chemistry of Phenothiazines" (Advances in heterocyclic chemistry, 1968, Vol. 9, pp. 321-460); B. Cardillo & G. Casnati in Tetrahedron, 1967, Vol. 23, p. 3771. Phenothiazine and phenoxazine propionic acids may be prepared as described in J.  
15 Org. Chem. 15, 1950, pp. 1125-1130. Hydroxyethyl and hydroxypropyl derivatives of phenothiazine and phenoxazine may be prepared as described by G. Cauquil in Bulletin de la Society Chimique de France, 1960, p. 1049.

- Methylsyningate, ethylsyningate, propylsyningate,  
20 butylsyningate, hexylsyningate and octylsyningate may be produced by using the method disclosed in Chem. Ber. 67, 1934, p. 67.

- Ethyl 3-(4-hydroxy-3,5-dimethoxyphenyl)acrylate was synthesised from syringaldehyde and triethyl phosphonoacetate in  
25 ethanol/sodium ethanolate. The product was after purification characterised by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR (showing spectra as expected) and the melting point was 68-70°C.

- The invention is further illustrated in the following examples which are not intended to be in any way limiting to the  
30 scope of the invention as claimed.

**EXAMPLE 1**Stain Bleaching with mediated laccase systems

5 To investigate the stain bleaching effect of mediated laccase systems, small-scale washing trials were carried out. Test pieces of fabric soiled with beta-carotene, curry, and paprika, respectively, were treated in beakers with magnetical stirring in borate solution with several combinations of laccases and  
10 mediators.

The reference treatment in each case was a wash under the same conditions with no laccase-mediator system present.

After the wash, the test pieces of fabric were thoroughly rinsed in cold tap water and air-dried in the dark  
15 overnight. The bleaching effect of any given laccase-mediator system was then evaluated by using a Datacolor Elrepho 2000 (remission measurements at 460 nm) or by using a Minolta Chroma Meter (colourspace XYZ) using D65 as the light source.

Positive values of more than 2-3 units indicate  
20 significant bleaching effects in both systems.

The mediators used are abbreviated as follows:

PPT: phenothiazine-10-propionic acid  
EPC: 4-carboxyphenothiazine-10-propionic acid  
25 POP: phenoxazine-10-propionic acid  
AS: acetosyreingone,  
(4-hydroxy-3,5-dimethoxyphenyl)ethan-1-one  
MS: methyl syringate,  
4-hydroxy-3,5-dimethoxybenzoic acid methyl ester

30

Wash trial 1. Laccase from *Trametes villosa*.

Medium: 25 mM borate (from boric acid with NaOH), pH 7.

Temperature: 40°C, 15°C, 10°C.

35 Duration of wash: 30 min.



Laccase preparation obtained in the following way:

800 ml culture broth of *Trametes villosa*, CBS 678.70, was filtered with filter aid to give a clear filtrate, which was concentrated and washed by ultrafiltration on a membrane with a cut-off of 6-8 kDa. One ml samples of concentrated preparation was applied onto a Q-Sepharose HP column (Pharmacia, Sweden) equilibrated with 0.1 M phosphate pH 7, and the laccase was eluted with a flat NaCl gradient around 0.25 M. Fractions with laccase activity from 10 runs were pooled and concentrated by ultrafiltration.

Laccase dosage: 1 mg/l washing liquor.

Mediator dosage: 120 µM. Mediators predissolved in ethanol.

Bleaching Results (40°C):

15

Delta (remission (%)) at 460 nm)

		<u>Betacarotene</u>	<u>Curry</u>	<u>Paprika</u>
20	PPT	14	6	22
	EPC	7	5	20
	POP	3	2	16
	AS	5	6	15
	MS	8	7	19

25

Bleaching results (15°C):

Calculated Delta-E (colourspace XYZ)

30

Paprika\*

PPT

18

Bleaching results (10°C):Calculated Delta-E (colourspace XYZ)

5

Paprika\*

PPT

21

10 \*) : The paprika test pieces of fabric were obtained from Center for Test materials, WFK P (P043).

It can be seen from the results presented above that paprika is bleached very effectively even at 10-15°C.

Wash trial 2. Laccase from *Coprinus cinereus*.

15

Medium: 25 mM borate (from boric acid with NaOH), pH 8.5.

Temperature: 40° C.

Duration of wash: 30 min.

Laccase preparation obtained in the following way:

20 *Coprinus cinereus* (IFO 30116 - freely available to the public from Institute of Fermentation, Osaka (IFO) under the indicated deposit number) was inoculated from a PDA agar slant (PDA: 39 g/l potato dextrose agar) into a 100 ml shake flask containing medium A (Medium A is described below). The culture was

25 cultivated for 6 days at 26°C and 100 rpm. A 10-liter fermentor containing medium A was inoculated with the 100 ml culture broth. The fermentation ran for 6 days at 26°C and 100 rpm. The culture broth was filtrated and concentrated by ultrafiltration.

30 Further purification was carried out using hydrophobic interaction chromatography followed by anionic exchange chromatography. This process resultated in at preparation with a laccase activity of 3.6 LACU/ml. The estimated purity was >80% on a protein basis.

Medium A:

Soja meal	30 g/l
Maltodextrin	15 g/l
bacto peptone	5 g/l
pluronic	0.2 g/l

5

Laccase dosage: 1 mg/l washing liquor.

Mediator dosage: 120  $\mu$ M. Mediators predissolved in ethanol.

Bleaching Results:

10

Delta (remission (%)) at 460 nm)

	<u>Betacarotene</u>	<u>Paprika</u>
15 PPT	16	5
EPC	12	8
AS	5	1
MS	15	6

20 Wash trial 3. Laccase from *Myceliophthora thermophila*.

Medium: 25 mM borate (from boric acid with NaOH) pH 8.5 at 40°C;  
25 mM borate (from boric acid with NaOH) pH 7 at 15°C and at  
10°C.

25 Temperature: 40°C, 15°C, 10°C.

Duration of wash: 30 min.

Laccase preparation obtained as described in PCT/US95/06815.

Laccase dosage: 1 mg/l washing liquor.

Mediator dosage: 120  $\mu$ M. Mediators predissolved in ethanol.

30

Bleaching Results (40°C):

		<u>Delta (remission (%)) at 460 nm)</u>	
		<u>Betacarotene</u>	<u>Paprika</u>
5	PPT	4	0
	EPC	3	3
	AS	7	4
10	MS	7	8

Bleaching results (15°C):

		<u>Calculated Delta-E (colourspace XYZ)</u>	
15			<u>Paprika*</u>
	MS	21	

20

Bleaching results (10°C):

		<u>Calculated Delta-E (colourspace XYZ)</u>	
25			<u>Paprika*</u>
	PPT	17	

30 \*) : The paprika test pieces of fabric were obtained from Center for Testmaterials, WFK P (P043).

It can be seen from the results presented above that paprika is bleached very effectively even at 10-15°C.

35

**EXAMPLE 2**Stain bleaching with mediated peroxidase systems

- 5 To investigate the stain bleaching effect of mediated peroxidase systems, a small-scale washing trial was carried out analogously to the ones described in Example 1.

Wash trial. Peroxidase from *Coprinus cinereus*.

10

Medium: 25 mM borate (from boric acid with NaOH), pH 7.

Temperature: 40°C, 15°C, 10°C.

Duration of wash: 30 min.

Peroxidase preparation obtained as described in WO 94/12621.

- 15 Peroxidase dosage: 0.2 mg/l washing liquor.

Mediator dosage: 120 µM. Mediators predissolved in ethanol.

Hydrogen peroxide: 0.5 mM.

Bleaching Results (40°C):

20

Delta (remission (%)) at 460 nm)

		<u>Betacarotene</u>	<u>Curry</u>	<u>Paprika</u>
25	PPT	12	9	25
	EPC	13	8	21
	POP	4	4	18
	AS	0	8	13
	MS	9	9	20

30

Bleaching results (15°C):Calculated Delta-E (colourspace XYZ)

35

Paprika\*

PPT

28

5 Bleaching Results (10°C):Calculated Delta-E (colourspace XYZ)

10

Paprika\*

MS

23

- 15 \*) : The paprika test pieces of fabric were obtained from Center for Testmaterials, WFK P (P043).

It can be seen from the results presented above that paprika is bleached very effectively even at 10-15°C.

20

**EXAMPLE 3**Stain bleaching with mediated peroxidase systems

- 25 Peroxidase-mediator systems identical to or similar to the ones studied in Example 2 were subjected to a Launder-ometer wash trial to investigate the effects of having a closed system and a different mechanical treatment. The Launder-ometer is a laboratory-scale set-up for simulating conditions in a traditional front-loaded European washing machine.

30

Wash trial. Peroxidase from *Coprinus cinereus*.

Medium: 25 mM borate (from boric acid with NaOH), pH 7.

Temperature: 40°C.

- 35 Duration of wash: 5 min preheating from 35°C to 40°C, then 30

min. wash at 40°C.

Peroxidase preparation obtained as described Example 2.

Peroxidase dosage: 120 µM. Mediators predissolved in ethanol.

Hydrogen peroxide: 0.5 mM.

5

Mediators tested: the above ones (see Example 1), except AS, and additionally MPT, 10-methylpenothiazine.

Bleaching results:

10

Delta (remission (%)) at 460 nm)

		<u>Betacarotene</u>	<u>Curry</u>	<u>Paprika</u>
15	PPT	11	6	11
	EPC	10	5	10
	MPT	8	4	6
	POP	6	3	10
	MS	7	7	10

20

It is seen that trends are much the same as in the small-scale washing test in Example 2. (The absolute numbers obtained depend on, among other things, wash load and mechanical action on the fabrics in the wash and are not directly comparable between the two examples here.)

25

#### EXAMPLE 4

Stain bleaching with a laccase and a series of homologous alkyl syringates as mediators

30

To investigate the effect on stain bleaching properties of homologous alkyl syringates mediators, a series of alkyl syringates was tested in an experiment much like the one in Example 3, except that the enzyme was a laccase instead of a

35

peroxidase.

Wash trial. Laccase from *Trametes villosa*.

Medium: 25 mM borate (from boric acid with NaOH), pH 7.

Temperature: 40°C.

- 5 Duration of wash: 5 min preheating from 35°C to 40°C, then 30 min wash at 40°C.

Laccase preparation obtained as described in Example 1.

Laccase dosage 1 mg/l washing liquor.

Mediator dosage: 120 µM. Mediators predissolved in ethanol.

10

Mediators tested: methyl, ethyl, propyl, butyl, hexyl, and octyl syringate.

Bleaching results:

15

Delta (remission (%)) at 460 nm)

	<u>Betacarotene</u>
methyl	13
20 ethyl	13
propyl	32
butyl	26
hexyl	8
octyl	2

25

It is seen that a marked optimum is reached at chain lengths 3 and 4 of the alkyl group. All these syringates except the octyl homologue achieve significant bleaching under the conditions given.



## CLAIMS

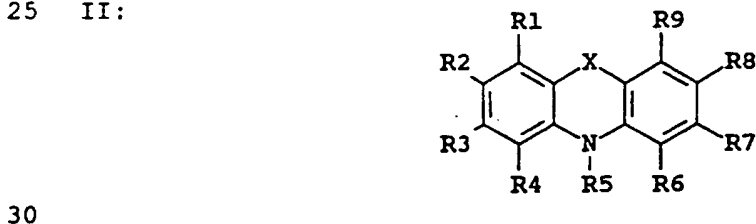
1. A process for bleaching stains present on fabric, comprising contacting, in an aqueous medium, the fabric with a phenol oxidizing enzyme system and a mediator for a sufficient period of time, wherein said mediator can be described by one of the following formulas:

I:



- in which formula A is a group such as  $-\text{CO}-\text{X}$ ,  $-\text{CH}=\text{CH}-\text{X}$ ,  $-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{X}$ ,  $-\text{CH}=\text{CH}-\text{CO}-\text{X}$ ,  $-\text{SO}_2-\text{X}$ ,  $-\text{PO}-\text{XZ}$ , in which X and Z may be identical or different and selected from the group consisting of  $-\text{H}$ ,  $-\text{OH}$ ,  $-\text{C}_n\text{H}_{2n+1}$ ,  $-\text{OC}_n\text{H}_{2n+1}$  and  $-\text{N}-\text{EF}$ , in which E and F may be identical or different and selected from the group consisting of  $-\text{H}$  and  $\text{C}_m\text{H}_{2m+1}$ ;  $1 \leq n \leq 5$ ; and B and C may be the same or different and selected from  $\text{C}_m\text{H}_{2m+1}$ ;  $1 \leq m \leq 5$ ; or

25 II:



- in which formula X represents  $(-\text{O}-)$  or  $(-\text{S}-)$ , and the substituent groups  $\text{R}^1-\text{R}^9$ , which may be identical or different, independently represents any of the following radicals:
- 35 hydrogen, halogen, hydroxy, formyl, carboxy, and esters and salts hereof, carbamoyl, sulfo, and esters and salts hereof, sulfamoyl, nitro, amino, phenyl,  $\text{C}_1-\text{C}_{14}$ -alkyl,  $\text{C}_1-\text{C}_5$ -alkoxy, carbonyl- $\text{C}_1-\text{C}_5$ -alkyl, aryl- $\text{C}_1-\text{C}_5$ -alkyl; which carbamoyl, sulfa-

moyl, and amino groups may furthermore be unsubstituted or substituted once or twice with a substituent group  $R^{10}$ ; and which phenyl may furthermore be unsubstituted or substituted with one or more substituent groups  $R^{10}$ ; and which  $C_1$ - $C_{14}$ -alkyl,  $C_1$ - $C_5$ -alkoxy, carbonyl- $C_1$ - $C_5$ -alkyl, and aryl- $C_1$ - $C_5$ -alkyl groups may be  
5 saturated or unsaturated, branched or unbranched, and may furthermore be unsubstituted or substituted with one or more substituent groups  $R^{10}$ ;

which substituent group  $R^{10}$  represents any of the  
10 following radicals: halogen, hydroxy, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, sulfamoyl, nitro, amino, phenyl, aminoalkyl, piperidino, piperazinyl, pyrrolidino,  $C_1$ - $C_5$ -alkyl,  $C_1$ - $C_5$ -alkoxy; which carbamoyl, sulfamoyl, and amino groups may furthermore be  
15 unsubstituted or substituted once or twice with hydroxy,  $C_1$ - $C_5$ -alkyl,  $C_1$ - $C_5$ -alkoxy; and which phenyl may furthermore be substituted with one or more of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts hereof, and sulfamoyl; and  
20 which  $C_1$ - $C_5$ -alkyl, and  $C_1$ - $C_5$ -alkoxy groups may furthermore be saturated or unsaturated, branched or unbranched, and may furthermore be substituted once or twice with any of the following radicals: halogen, hydroxy, amino, formyl, carboxy and esters and salts hereof, carbamoyl, sulfo and esters and salts  
25 hereof, and sulfamoyl;

or in which general formula two of the substituent groups  $R^1$ - $R^9$  may together form a group -B-, in which B represents any of the following the groups:  $(-CHR^{10}-N=N-)$ ,  $(-CH=CH-)_n$ ,  $(-CH=N-)_n$  or  $(-N=CR^{10}-NR^{11}-)$ , in which groups n represents an  
30 integer of from 1 to 3,  $R^{10}$  is a substituent group as defined above and  $R^{11}$  is defined as  $R^{10}$ .

2. A process according to claim 1, in which the phenol oxidizing enzyme system is a peroxidase and a hydrogen peroxide source.

3. A process according to claim 2, wherein the peroxidase is horseradish peroxidase, soybean peroxidase or a peroxidase enzyme derived from *Coprinus*, *Bacillus* or *Myxococcus*.
- 5 4. A process according to claim 2 or 3, wherein the hydrogen peroxide source is hydrogen peroxide or a hydrogen peroxide precursor.
- 10 5. A process according to claim 1, wherein the aqueous medium contains  $H_2O_2$  or a precursor for  $H_2O_2$  in a concentration corresponding to 0.001-25 mM  $H_2O_2$ .
- 15 6. A process according to claim 1, in which the phenol oxidizing enzyme system is a laccase or a laccase related enzyme together with oxygen.
7. A process according to claim 6, wherein the laccase is derived from *Trametes*, *Coprinus*, or *Myceliophthora*.
- 20 8. A process according to claim 1, wherein the concentration of the phenol oxidizing enzyme corresponds to 0.001-100 mg of enzyme protein per liter of aqueous medium.
- 25 9. A process according to claim 1, wherein the mediator belongs to the group consisting of acetosyringone, syringaldehyde, methylsyringate and syringic acid.
- 30 10. A process according to claim 1, wherein the mediator belongs to the group consisting of 10-methylphenothiazine, phenothiazine-10-propionic acid, phenoxazine-10-propionic acid, phenoxazine-10-hydroxyethyl, phenothiazine-10-ethyl-4-carboxy, promazine hydrochloride and phenothiazine-10-ethylalcohol.
- 35 11. A process according to claim 1, wherein the mediator in the aqueous medium is present in concentrations of from 0.01 to 5000  $\mu M$ .

12. A process according to claim 1, wherein the phenol oxidizing enzyme system in the aqueous medium is present in concentrations of from 0.001-100 mg of enzyme protein per liter.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 96/00390

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: D06L 3/02, C11D 3/386

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: D06L, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, IFIPAT, CA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9412621 A1 (NOVO NORDISK A/S), 9 June 1994 (09.06.94)	1-12
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P,X	WO 9612845 A1 (NOVO NORDISK A/S), 2 May 1996 (02.05.96)	1-12
	---	
P,X	WO 9612846 A1 (NOVO NORDISK A/S), 2 May 1996 (02.05.96)	1-12
	---	
A	WO 9218683 A1 (NOVO NORDISK A/S), 29 October 1992 (29.10.92)	1-12
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☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

28/10/96

International application No.  
PCT/DK 96/00390

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WO-A1- 9412621	09/06/94	CA-A- 2150562 EP-A- 0679183 FI-A- 952648 JP-T- 8506009 AU-A- 7937194 CA-A- 2175047 EP-A- 0730641 WO-A- 9511964 CA-A- 2150563 EP-A- 0677102 FI-A- 952647 JP-T- 8503371 WO-A- 9412620	09/06/94 02/11/95 28/07/95 02/07/96 22/05/95 04/05/95 11/09/96 04/05/95 09/06/94 18/10/95 31/05/95 16/04/96 09/06/94
WO-A1- 9612845	02/05/96	AU-A- 3650195	15/05/96
WO-A1- 9612846	02/05/96	AU-A- 3650295	15/05/96
WO-A1- 9218683	29/10/92	NONE	